

# Wood smoke particles generate free radicals and cause lipid peroxidation, DNA damage, NFκB activation and TNF-α release in macrophages

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## Abstract

The present study investigated the generation of free radicals by wood smoke and cellular injuries caused by these radicals. Wood smoke was collected after thermolysis of western bark. Electron spin resonance (ESR) techniques were used to measure both carbon-centered radicals and generation of reactive oxygen species (ROS) by wood smoke. Wood smoke, in the presence of H<sub>2</sub>O<sub>2</sub>, was found to be able to generate hydroxyl radical (<sup>•</sup>OH). DNA strand breakage was measured by exposing wood smoke to λ Hind III fragments using gel electrophoresis. Wood smoke combined with H<sub>2</sub>O<sub>2</sub> caused DNA damage. Sodium formate, an <sup>•</sup>OH radical scavenger, or deferoxamine, a metal chelator, inhibited the DNA damage. Cellular DNA damage was also measured in cultured RAW 264.7 mouse macrophage cells by the single cell gel (SCG) electrophoresis assay. Cells were exposed to wood smoke samples for various times and significant DNA damage was observed. Elemental analysis was performed on the filter samples and the presence of Fe was noteworthy. Wood smoke is also able to cause lipid peroxidation, activate nuclear transcription factor, NFκB, and enhance the release of TNF-α from RAW 264.7 cells. The results indicate that the free radicals generated by wood smoke through the reaction of Fe with H<sub>2</sub>O<sub>2</sub> are able to cause DNA and cellular damage and may act as a fibrogenic agent. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Wood smoke; Free radicals; DNA damage

## 1. Introduction

Wood smoke has been studied as part of hazard evaluation for exposure from both structural fires and home wood burning stoves (Jankovic et al., 1993; Larson and Koenig, 1994). Hazards

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encountered by fire fighters can include both physical, respiratory and systemic health dangers. These exposures are created from inhalation of by-products and intermediates of the combustion process.

Fire fighter exposure can occur during two separate types of activities, knockdown and overhaul. Knockdown involves activities associated with extinguishing structural fires. Overhaul consists of preventing re-ignition of the fire from isolated hot spots. A study by the National Institute for Occupational Safety and Health reported the presence of environmental contaminants during knockdown and overhauling of actual fires (Jankovic et al., 1991).

The use of wood burning stoves as a alternate heating source has increased in recent years. Wood burning has been an attractive source of home heating in part because it uses a renewable resource as fuel. However, this is offset by air pollution emissions from these stoves. Legislation restricting the sale and types of stoves has reduced emissions in modern wood stoves but many older models are still in use. A number of communities experience elevated levels of wood smoke during the winter as well as high indoor levels of air pollution in homes with poorly functioning stoves.

Combustion is defined as a self-sustaining oxidation process which involves the reaction of fuel (reducing agent) and oxygen (oxidizing agent) which is initiated by activating energy (heat). This chemical reaction produces more energy and causes a chemical chain reaction. The chemical intermediates generated during combustion are usually in the form of hydrogen, carbon and oxygen free radicals (Tuve, 1976). Measurement of long-lived free radicals and their ability to be absorbed and initiate toxic reactions in the pulmonary tract has also been conducted (Lachoki et al., 1989; Chemical and Engineering News, 1990; Pryor et al., 1990). Previous investigations have identified wood smoke as a source of free radicals (Lowry et al., 1985; Yamaguchi et al., 1992). Potential adverse health effects from these exposures have become a topic of interest recently.

Earlier studies (Brown, et al., 1988a; Kimura et al., 1988; Traber et al., 1989) have suggested a

role of free radicals in smoke inhalation injury but have not identified the types of free radicals involved or the biological mechanism of action. The present investigation will attempt: (a) to detect and identify the free radicals associated with wood smoke; (b) to examine the ability of wood smoke to cause specific DNA damage and activation of cellular responses involving TNF- $\alpha$  and NF $\kappa$ B; (c) to analyze the ability of wood smoke to cause lipid peroxidation in macrophage cells; and (d) to define the role of free radical reactions involved in these processes.

## 2. Materials and methods

### 2.1. Reagents

Deferoxamine, 5,5-dimethyl-1-pyrroline-oxide (DMPO), H<sub>2</sub>O<sub>2</sub>, and sodium formate were purchased from Sigma Chemical Company (St. Louis, MO). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation and was free of ESR detectable impurities. Phosphate buffered saline (PBS), (KH<sub>2</sub>PO<sub>4</sub> (1.06 mM), Na<sub>2</sub>HPO<sub>4</sub> (5.6 mM), NaCl (154 mM), pH 7.4), was purchased from BioWhittaker Inc. (Walkersville, MD). Chelex 100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA). The PBS was treated with chelex 100 to remove transition metal ion contaminants.

### 2.2. Cell culture

The mouse peritoneal macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC) (Rockville, MD). RAW 264.7 cells are commonly used and have been found to respond to particle exposure (transcription factor activation and TNF- $\alpha$  production) in a manner similar to primary alveolar macrophages (data from our lab). The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 1000 units/ml penicillin-streptomycin. (Sigma Chem Co.)

### 2.3. Sample collection and preparation

Smoke was generated by thermolysis of 100 g of western bark (pine and fir) in a crucible furnace (Lindberg, Waterton, WI) at 400°C. The smoke was delivered into a 20 l reservoir and mixed 1:1 with 100% oxygen. Smoke exiting the system was filtered for 1 min through a Whatman 1 filter at 0, 5, 10, 15 and 20 min after initiating the flow of smoke. In other experiments, at the above times, liquid samples were prepared by bubbling the smoke through 10 ml of sterile saline for 1 min. Additional smoke samples were passed through 37 mm nitrocellulose filters for elemental analysis. The filters or saline were frozen in dry ice until use.

Filter samples were used in ESR measurement of carbon-centered radicals and elemental analysis. While the liquid samples were used in the ESR spin trapping measurements, DNA damage, single cell gel analysis, lipid peroxidation, TNF- $\alpha$ , and NF $\kappa$ B measurements.

### 2.4. Free radical measurements

For detection and identification of short-lived radicals, a spin trapping method was used. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splitting of the spin adduct is generally characteristic of the original, short-lived, trapped radical. This method is specific and sensitive and is considered to be the method of choice for the detection and identification of free radicals.

All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate ( $K_3CrO_8$ ) and 1,1 diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the

peak height by  $(DH_{pp})^2$ , where  $DH_{pp}$  represents peak to peak width. An EPRDAP 2.0 program (U.S. EPR, Inc. Clarksville, MD) was used for data acquisition and analysis. All experiments were performed at room temperature and under ambient air. Filter measurements were performed on exposed Whatman 1 filters which were placed into a quartz sample tube and then inserted into the ESR sample cavity for measurement. Liquid samples were mixed with reactants to a final volume of 1.0 ml (100  $\mu$ l DMPO (1 M), 100  $\mu$ l  $H_2O_2$  (10 mM), 100  $\mu$ l wood smoke sample and 700  $\mu$ l PBS). The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations.

### 2.5. DNA strand breakage

The DNA strand breakage assay was performed in PBS (pH 7.4) in 1.5 ml polypropylene tubes at 37°C. A reaction mixture contained 10 mg DNA (1 Hind III digest) in a total volume of 100  $\mu$ l of buffer. To this solution, 2 ml of gel loading buffer (50 mM EDTA, 25% sodium dodecyl sulfate (SDS) and 0.1% bromophenol blue) were added. Wood smoke exposed samples contained 100  $\mu$ l liquid wood smoke sample and were incubated for 30 min. The samples were then electrophoresed in 0.7% agarose at 1–2 V/cm in 40 mM Tris–acetate buffer containing 2 mM EDTA (pH 8.0). Gels were stained in ethidium bromide (5 mg/ml) for 20 min and scanned under UV light using a Stratagene Eagleeye II (La Jolla, CA).

### 2.6. Single cell gel electrophoresis assay (SCG assay) for DNA damage

Cellular DNA damage was determined by the SCG assay described below. The tail length was utilized as the measurement of DNA damage; the longer the tail, the more severe the damage. RAW 264.7 macrophage cells ( $2.5 \times 10^6$ /ml) were seeded in each well of a six-well plate and incubated at 37°C under 5%  $CO_2$  overnight. The cells were exposed to 50  $\mu$ l of liquid wood smoke samples. After 24 h exposure, the cells were har-

vested and re-suspended in PBS at an approximate density of  $2 \times 10^6$ /ml for SCG assay.

The SCG assay was performed according to the method reported previously (Tice et al., 1991) with minor modification. One hundred microlitres of 0.5% normal melting agarose (Sigma) in PBS was added onto the fully frosted microscope slides, then covered with a  $22 \times 22$  mm coverslip immediately. Slides were then placed on ice for 10 min. One hundred microlitres of cell suspension was mixed with 900  $\mu$ l of low melting point (LMP) agarose (Sigma) at  $37^\circ\text{C}$ , then 75  $\mu$ l of the mixture (approximate  $1.5\text{--}2.0 \times 10^4$  cells) was pipetted onto the first agarose layer (after gentle removal of the coverslip). The coverslip was replaced and the slide was maintained at  $4^\circ\text{C}$  for solidification. After removal of the coverslip, 75  $\mu$ l of 0.5% LMP agarose was loaded, the coverslip was replaced, and the gel was allowed to solidify for 10 min. The coverslip was removed and the slides were immersed in freshly prepared cold lysing solution (2.5 mM NaCl, 100 mM  $\text{Na}_2\text{EDTA}$ , 10 mM Tris, and 1% sodium sarcosinate, pH 10; 1% Triton X-100 and 10% DMSO v/v were added just before use) for at least 1 h at  $4^\circ\text{C}$ . All the following steps were conducted under red light to prevent additional DNA damage. The slides were drained and placed in a horizontal gel electrophoresis tray filled with fresh alkaline-EDTA buffer (300 mM NaOH and 1 mM EDTA in distilled water, pH  $> 13$ ) for 10 min to allow for DNA unwinding and alkaline-labile damage expression. Electrophoresis was carried out for 50 min at room temperature at 20 V (about 1 V/cm). Then the slides were neutralized by rinsing for 5 min with Tris buffer (0.4 M Tris, pH 7.5) three times. Slides then were stained with 100  $\mu$ l ethidium bromide (2  $\mu\text{g}/\text{ml}$ ) and covered with a coverslip. Image analysis was made at  $400\times$  magnification using a fluorescence microscope (Zeiss microscopes, Thornwood, NY). The length of DNA migration was determined with an optical micrometer by measuring the tail length (TL, distance between the edge of head and the end of tail) in microns. A minimum of 50 cells per treatment, 25 cells on each of two duplicate slides from a randomly chosen field, was scored.

## 2.7. Elemental analysis

Elemental analysis of filter smoke samples was conducted by NIOSH method 7300 (NMAM fourth edition, 8/15/94). Filter samples were prepared as follows. Each sample was translated to a clean microwave digestive vessel. A 10 ml aliquot of 1:1 nitric acid and American Society for Testing and Materials (ASTM) type II  $\text{H}_2\text{O}$  was added to each of the samples. The vessels were sealed and placed in the microwave oven for digestion. After the digestion, the samples were diluted to a final volume in 25 ml volumetric flasks with ASTM type II  $\text{H}_2\text{O}$ . Samples were transferred to dilution vials prior to analysis. The samples were analyzed using a Fisons ACCURIS inductively coupled plasma emission spectrometer controlled by a digital DEC station 466D2LP personal computer. Elemental analyses were performed by Data Chem Laboratories Inc. (Salt Lake City, UT).

## 2.8. Lipid peroxidation measurements

Lipid peroxidation of RAW 264.7 mouse monocytes was measured by monitoring the thiobarbituric acid (TBA) reactive substances formed in a reaction mixture, according to the method described by Hunter et al. (1963). A typical reaction mixture contained 100  $\mu$ l of liquid wood smoke sample and  $1 \times 10^7$  cells in a total volume of 1.0 ml PBS (pH 7.4). The mixture was exposed for 1 h in a shaking water bath at  $37^\circ\text{C}$ . The reaction was terminated by the addition of 0.625 ml of 40% trichloroacetic acid and 0.3 ml of 5 N hydrochloric acid. Vials were vortexed for 10 s and 0.625 ml of 2% thiobarbituric acid was added and mixed again. The mixture was then heated for 20 min at  $100^\circ\text{C}$ . The tubes were cooled and centrifuged for 10 min at 600 g. The absorbance of the supernate was measured at 585 nm. Malondialdehyde standards were prepared from 1,1,3,3,-tetramethoxypropane for a calibration curve, which was used to calculate the amount of TBA reactive substances produced by smoke samples. The concentrations provided in figure legends are final concentrations. All the experiments were carried out in air at room temperature.

## 2.9. Nuclear extraction

The nuclear extracts were prepared according to the modified method of Sun (Sun et al., 1994). Briefly,  $2 \times 10^6$  of RAW 264.7 cells were exposed to 100  $\mu$ l/ml of liquid wood smoke for 12 or 24 h then treated with 500  $\mu$ l of lysis buffer (50 mM KCl, 0.5% NP-40, 25 mM HEPES pH 7.8, 1 mM PMSF, 10 mg/ml leupeptin, 20 mg/ml aprotinin, and 100 mM DTT) on ice for 4 min. After a 1 min centrifugation at 14 000 rpm, the supernatant was saved as a cytoplasmic extract. The nuclei were washed once with the same volume of buffer without NP-40, then were added to a 300  $\mu$ l volume of extraction buffer (500 mM KCl, 10% glycerol with the same concentration of HEPES, PMSF, leupeptin, aprotinin and DTT as the lysis buffer) and pipetted several times. After centrifugation at 14 000 rpm for 5 min, the supernate was collected as the nuclear protein extract and stored at  $-70^\circ\text{C}$ . The protein concentration was determined by BCA (Pierce, Rockford, IL)

## 2.10. Electrophoresis mobility shift assay

The DNA–NF- $\kappa$ B binding reaction was conducted in a 24  $\mu$ l reaction mixture containing 1 mg Poly dI.dC (Sigma), 3  $\mu$ g nuclear protein extract, 3 mg BSA,  $4 \times 10^4$  cpm of  $^{32}\text{P}$ -labeled oligonucleotide probe and 12  $\mu$ l of  $2 \times \text{Y}$  buffer (24% glycerol, 24 mM HEPES pH 7.9, 8 mM Tris–HCl pH 7.9, 2 mM EDTA, and 2 mM DTT). This mixture was incubated on ice for 10

min in the absence of the radio-labelled probe, then incubated for 20 min at room temperature in the presence of radio-labelled probe. After incubation, the DNA-protein complexes were resolved on a 5% acrylamide gel (National Diagnostics, Atlanta, GA) that had been pre-run at 110 V for 1 h with  $0.5 \times \text{Tris}$ –boric acid–EDTA buffer. The loaded gel was run at 210 V for 90 min, then dried and placed on Kodak X-Omat film (Eastman Kodak, Rochester, NY) for autoradiography. The film was developed after overnight exposure at  $-70^\circ\text{C}$ .

## 2.11. TNF- $\alpha$ assay

Mouse macrophage cells (RAW 264.7) were cultured at  $5 \times 10^6$ /ml in DMEM medium with 10% FBS in a 6-well tissue culture plate. After 24 h of incubation, 100  $\mu$ l of liquid wood smoke sample were added to specific wells. Cells were exposed to wood smoke for and additional 12 h. TNF- $\alpha$  released to the media by the cells was determined using a TNF- $\alpha$  ELISA kit according to the manufacture's instruction (Endogen, Cambridge, MA). All experiments were carried out in air at room temperature except those specifically indicated. Data are presented as means  $\pm$  standard deviations for  $n$  experiments. Statistics were performed using a Student's  $t$ -test with significance set at  $P < 0.05$ .

## 3. Results

### 3.1. Stable free radicals generated by wood smoke

Fig. 1 shows a typical ESR spectrum recorded from a wood smoke filter sample. The spectrum was identified as carbon-centered radicals ( $\text{R}^\bullet$ ) based on the spectral line shape and position. These are relatively stable free radicals with a half-life of several days depending on the environmental conditions, such as temperature. When stored at room temperature, no change in the signal intensity was observed from wood smoke filter samples stored for three days (data not shown). Carbon-centered radicals were not observed when filters were in an aqueous solution.

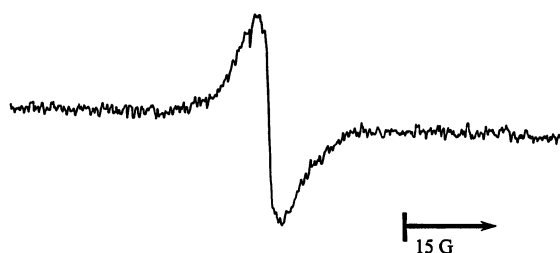


Fig. 1. A typical ESR spectrum recorded from a dry filter measurement. No change in spectral intensity was observed over a 3 day period. ESR settings were: center field, 3385 G; scan width, 100 G; time constant, 0.25 s; modulation amplitude, 1 G; receiver gain,  $2.5 \times 10^4$ ; frequency, 9.425 Gz; and power, 50 mW.

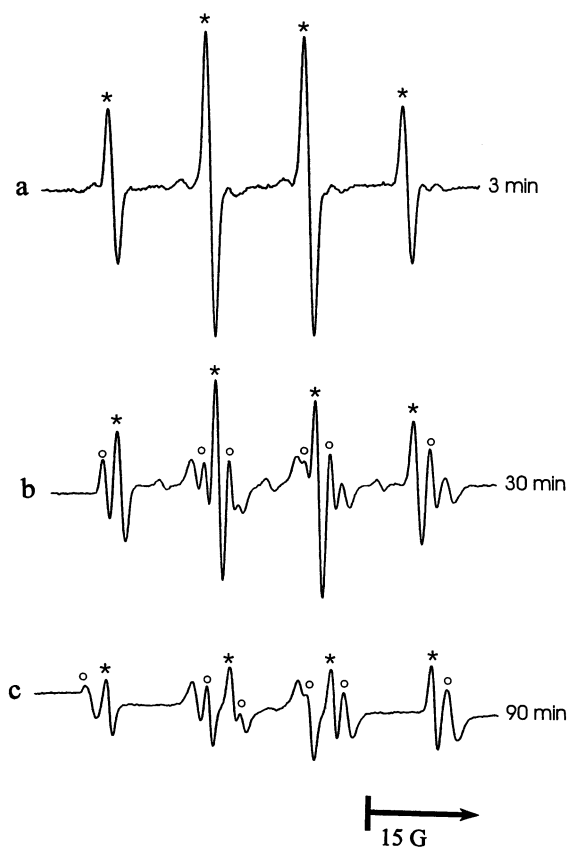


Fig. 2. The generation of short-lived  $\cdot\text{OH}$  radicals (\*) and reactive carbon-centered radicals (o) by wood smoke upon reaction with  $\text{H}_2\text{O}_2$ . (a) ESR spectrum recorded 3 min after reaction was initiated in PBS (pH 7.4) containing 10  $\mu\text{l}/\text{ml}$  wood smoke, 1 mM  $\text{H}_2\text{O}_2$  and 100 mM DMPO. (b) Same as (a) but the spectrum was recorded 30 min after mixing the reactants. (c) Same as (a) but the spectrum was recorded 90 min after mixing the reactants. Note that at the earlier stage of the reaction  $\cdot\text{OH}$  radical generation became was predominant, while at a later stage reactive carbon-centered radical generation predominant. ESR settings were: center field, 3385 G; scan width, 100 G; time constant, 0.25 sec; modulation amplitude, 1 G; receiver gain,  $2.5 \times 10^4$ ; frequency, 9.424 GHz; and power, 50 mW.

Wood smoke filters with added DMPO showed no radicals. Several types of filters were tested in the initial stages of the investigation. The filter type did not effect the radicals. Control filters, which had 100%  $\text{O}_2$  passed through them, showed no radical formation.

### 3.2. Reactive free radicals generated by wood smoke upon reaction with $\text{H}_2\text{O}_2$

Filters were treated with  $\text{H}_2\text{O}_2$  in the presence of the spin trap DMPO to measure their ability to generate reactive radical species. Fig. 2a shows a typical ESR spectrum. This spectrum exhibits a 1:2:2:1 quartet with hyperfine splittings of  $a_{\text{N}} = a_{\text{H}} = 14.9$  G. Based on these splitting constants, this 1:2:2:1 quartet was assigned to the DMPO/ $\cdot\text{OH}$  adduct as evidence for  $\cdot\text{OH}$  generation. It may be noted from Fig. 2 that initially, the radicals produced are predominantly  $\cdot\text{OH}$  with a small amount of reactive carbon-centered radicals. Over time (3–90 min) the relative proportion of  $\cdot\text{OH}$  to carbon-centered radicals decreased. It appears that the generation of  $\cdot\text{OH}$  radicals is predominant in the initial phase and that of reactive carbon centered radicals is predominant in the later phase. Liquid wood smoke samples were also treated with  $\text{H}_2\text{O}_2$  and DMPO to trap  $\cdot\text{OH}$  radicals in these samples. All liquid wood smoke samples showed generation of  $\cdot\text{OH}$  radicals (data not shown). Control samples of blank filters, DMPO,  $\text{H}_2\text{O}_2$  or combinations of the three showed no radical generation. No significant difference ( $P < 0.05$ ) was observed among wood smoke samples collected at different times during the combustion process.

### 3.3. Wood smoke induced DNA damage

As shown in Fig. 3, DNA strand breaks were not observable with DNA alone (lane 1) or DNA plus  $\text{H}_2\text{O}_2$  (lane 2). However, wood smoke caused observable DNA damage (lane 3). Addition of  $\text{H}_2\text{O}_2$  to the wood smoke sample intensified the DNA damage (lane 4). Both sodium formate, an  $\cdot\text{OH}$  radical scavenger, and deferoxamine, a metal chelator, inhibited DNA damage caused by wood smoke plus  $\text{H}_2\text{O}_2$  (lanes 5 and 6, respectively) and completely blocked DNA damage from wood smoke in the absence of  $\text{H}_2\text{O}_2$  (data not shown). The DNA damage was seen in wood smoke from all collection times.

Cellular DNA damage induced by wood smoke samples was measured by the SCG assay as described in the Materials and Methods. The tail

length was utilized as the measurement of DNA damage, the longer the tail, the more severe the damage. Wood smoke samples were collected at various times during the combustion process. As shown in Fig. 4, wood smoke samples collected at 15 and 20 min after ignition of the fire exhib-

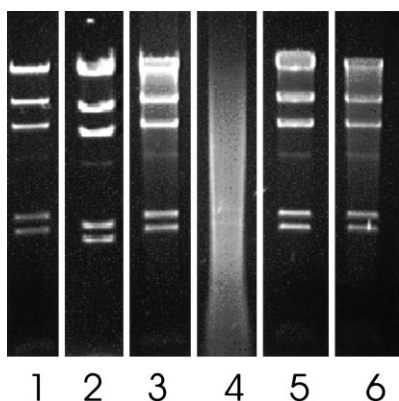


Fig. 3. DNA strand breaks induced by wood smoke. Lane 1,  $\lambda$  Hind III digested DNA alone; Lane 2, DNA +  $H_2O_2$  (1 mM); Lane 3, DNA + wood smoke (100  $\mu$ l/ml); Lane 4, DNA + wood smoke (100  $\mu$ l/ml) +  $H_2O_2$  (1 mM); Lane 5, DNA + wood smoke (100  $\mu$ l/ml) +  $H_2O_2$  (1 mM) + sodium formate (25 mM); Lane 6, DNA + wood smoke (100  $\mu$ l/ml) +  $H_2O_2$  (1 mM) + deferoxamine (2 mM).

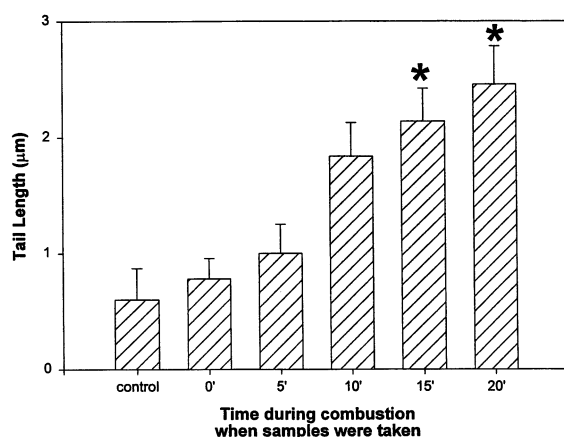


Fig. 4. DNA damage in macrophages induced by wood smoke as measured by the single cell electrophoresis assay. An increase in tail length represents increased DNA damage. Data presented are means of  $\pm$  S.D. for three sets of experiments. Asterisks indicate a significant increase in DNA damage compared to control cells. ( $P < 0.05$ ).

Table 1

Elemental analysis of wood smoke filters

Element	Limit of detection ( $\mu$ g/filter)	Amount detected ( $\mu$ g/filter)
Cobalt	0.2	ND
Chromium	0.5	ND
Iron	0.4	$1.58 \pm 0.53^a$
Nickel	0.3	ND

<sup>a</sup> Average of ten filters. ND, not detectable.

ited significant enhancement in cellular DNA damage.

### 3.4. Elemental analysis

It is well established that transition metals are involved in  $\bullet OH$  generation from  $H_2O_2$ . The mechanism involves metal-mediated Fenton or Fenton-like reactions as described in the following reaction  $[M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \bullet OH + OH^-]$ , where  $M^{n+}$  is the transition metal ion and  $M^{(n+1)+}$  is at a higher oxidation state. These transition metals include Co, Cr, Fe and Ni. The presence of these metals in the wood smoke was examined by elemental analysis. As shown in Table 1, Fe is the only transition metal present in a measurable amount.

### 3.5. Wood smoke-induced lipid peroxidation

Previous studies (Vallyathan et al., 1988, 1992) have shown that  $\bullet OH$  radical was able to induce lipid peroxidation. In this study, we examined whether wood smoke can cause lipid peroxidation. As shown in Fig. 5, wood smoke samples collected at various times during combustion substantially increased the amount of lipid peroxidation over controls in RAW 264.7 cells. At the 20 min collection time, wood smoke increased lipid peroxidation 2.9-fold above control.

### 3.6. Induction of $TNF-\alpha$ by wood smoke

The induction of  $TNF-\alpha$  by wood smoke was examined using RAW 264.7 cells exposed to 100  $\mu$ l/ml wood smoke for 12 or 24 h, respectively.

TNF- $\alpha$  secretion was determined by a TNF- $\alpha$  ELISA kit. The results in Fig. 6 show that wood smoke caused a significant increase in TNF- $\alpha$  activity; i.e. exposure of macrophages to a wood

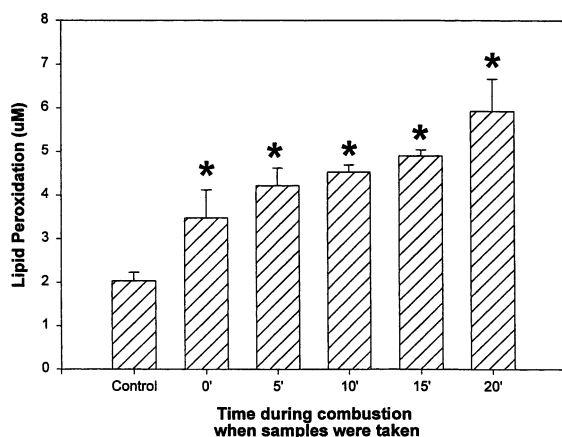


Fig. 5. Wood smoke induced lipid peroxidation. Exposure mixture contained 100  $\mu$ l wood smoke sample and  $5 \times 10^7$  RAW 264.7 cells. Data presented are means of  $\pm$  S.D. for four sets of experiments. Asterisks indicate a significant increase in lipid peroxidation compared to control ( $P < 0.05$ ).

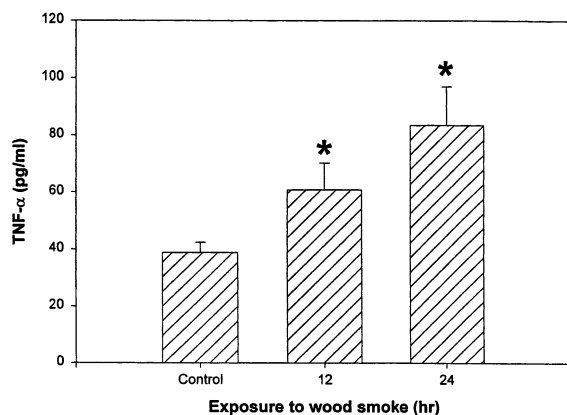


Fig. 6. Effect of exposure to wood smoke on TNF- $\alpha$  production by RAW 264.7 cells. The RAW 264.7 cells were cultured at  $5 \times 10^6$ /ml DMEM medium supplemented with 10% FBS in 6-well tissue culture plates. After 48 h incubation, the cells were stimulated with 100  $\mu$ l/ml wood smoke solution. The cells were then exposed for an additional 12 or 24 h. TNF- $\alpha$  release by macrophages was determined using a TNF- $\alpha$  ELISA kit. Values are means of  $\pm$  S.D. of 4 replicates. Asterisks indicate a significant increase compared to control ( $P < 0.05$ ).

## Induced NF $\kappa$ B activation

smoking sample ( $\mu$ l) 0 10 20

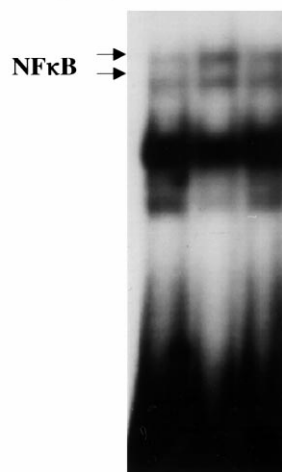


Fig. 7. Effect of wood smoke on NF $\kappa$ B activation in RAW 264.7 cells. Macrophages ( $5 \times 10^6$ /ml) were exposed to 0, 10 or 20  $\mu$ l/ml wood smoke, then subjected to extraction of nuclear proteins as described in the Materials and Methods. DNA binding activity of NF $\kappa$ B protein was detected with a  $^{32}$ P labelled probe double strand NF $\kappa$ B oligonucleotide by the electrophoresis mobility shift assay. Lane 1, untreated cells; lane 2, cells + 10  $\mu$ l/ml wood smoke; lane 3, cells + 20  $\mu$ l/ml wood smoke.

smoke solution for 24 h increased TNF- $\alpha$  production by 2.1-fold.

### 3.7. NF $\kappa$ B activation by wood smoke

Previous studies have shown that induction of NF $\kappa$ B activity may be responsible for TNF- $\alpha$  gene transcription (Ye et al., 1999). Therefore, RAW 264.7 cells were exposed to wood smoke solutions and NF $\kappa$ B activation was monitored. The cells were incubated in the presence of 10 or 20  $\mu$ l/ml of wood smoke and then harvested for extraction of nuclear proteins. These nuclear proteins were analyzed using the electrophoretic mobility shift assay (EMSA) to determine DNA binding activity of NF $\kappa$ B. As shown in Fig. 7, the untreated RAW 264.7 cells (lane 1), exhibited a low level of NF $\kappa$ B activity. After treatment with wood smoke, 10 and 20  $\mu$ l/ml, NF $\kappa$ B activation was observed (lanes 2 and 3, respectively).

#### 4. Discussion

The results obtained in the present study show that wood smoke is able to generate stable carbon-centered radicals. Upon reaction with  $\text{H}_2\text{O}_2$ , wood smoke produced predominantly  $\cdot\text{OH}$  radicals initially, while reactive carbon-centered radicals became more apparent later. In the presence of  $\text{H}_2\text{O}_2$ , wood smoke caused DNA damage. The major species responsible for DNA damage is  $\cdot\text{OH}$  radical. The following experimental observations support this conclusion. (a) Sodium formate, a scavenger of  $\cdot\text{OH}$  radical, protected the DNA from damage. (b) Deferoxamine, which reduced the ability of metal ions to generate  $\cdot\text{OH}$  from  $\text{H}_2\text{O}_2$ , inhibited DNA damage. (c) ESR measurements show that  $\cdot\text{OH}$  radical is generated upon reaction of wood smoke with  $\text{H}_2\text{O}_2$ . (d) Metal ions, such as  $\text{Fe(II)}$ , are present in wood smoke as detected by elemental analysis. The reaction of  $\text{Fe(II)}$  with  $\text{H}_2\text{O}_2$  through the Fenton reaction may be the mechanism responsible for  $\cdot\text{OH}$  generation. The wood smoke induced  $\cdot\text{OH}$  generation could be particularly significant during phagocytosis (upon exposure to wood smoke). In this process alveolar macrophages and other cellular constituents generate large amount of  $\text{H}_2\text{O}_2$  as part of the respiratory burst. The  $\text{H}_2\text{O}_2$  would react with  $\text{Fe(II)}$  present in the wood smoke to generate  $\cdot\text{OH}$  radicals.

The results obtained from the present study demonstrate that wood smoke is capable of causing lipid peroxidation.  $\cdot\text{OH}$  radicals have been shown to cause cell membrane damage and initiate lipid peroxidation (Shi et al., 1989). Lipid peroxidation would result in the release of lipid-derived radicals ( $\text{R}\cdot$ ,  $\text{RO}\cdot$ , and  $\text{ROO}\cdot$ ) (Halliwell and Gutteridge, 1985; Shi et al., 1988). These reactive species could further react with the cell membrane, leading to additional damage. Damage to cell membrane may lead to increased release of catalytically active iron (Vladimirov, 1986), which would increase generation of ROS further. For example, it has been reported (Morita et al., 1983; Ueda et al., 1985) that incubation of autoxidized linoleic acid induced DNA strand breakage, but only to a minor extent. When metal ions such as  $\text{Fe(II)}$  were added, DNA damage

increased. The products of lipid peroxidation, malondialdehyde and other groups of aldehyde products, such as hexanal, 4-hydroxynonenal and aldehydes, may also cause DNA damage (Esterbauer, 1982; Vaca et al., 1988). It has been proposed that free radicals derived from lipid peroxidation may function as tumor initiators (Comporti, 1985; Vaca et al., 1988; Brambilla et al., 1989). It thus appears that Fe presented in wood smoke may be considered as an indirect tumor initiator.

The results obtained from the present study also show that wood smoke is able to activate  $\text{NF}\kappa\text{B}$ . This transcription factor is found in many different cell types and is a focal point for understanding how extracellular signals induce the expression of specific sets of early response genes in higher eukaryotes. This transcription factor is critical to the expression of many cytokines involved in inflammation, including interleukin-1 (IL-1), IL-6,  $\text{TNF-}\alpha$ , and chemokines.  $\text{NF}\kappa\text{B}$  can be induced by oxidative stress (Ye et al., 1999). A recent study has shown that among ROS,  $\cdot\text{OH}$  radical functions as a messenger for  $\text{NF}\kappa\text{B}$  activation (Shi et al., 1999). Other studies have reported that  $\text{NF}\kappa\text{B}$  activation is important in cells signalling upon exposure to injury-producing conditions (Baeurle, 1991).  $\text{NF}\kappa\text{B}$  serves as a second messenger to induce a series of cellular cytokines in response to a disruption in the cell's environment (Osborn et al., 1989).  $\text{NF}\kappa\text{B}$  activates these cytokines by acting as a transcriptional factor and binding to  $\text{NF}\kappa\text{B}$  promoter sequences. Indeed, the present study shows that wood smoke is a potent inducer of  $\text{TNF-}\alpha$  release. This cytokine is a macrophage-derived peptide that has been shown to play an important role in the pathogenesis of pulmonary fibrosis (Driscoll et al., 1990). Several fibrogenic agents, such as silica and asbestos, stimulate  $\text{TNF-}\alpha$  mRNA expression and protein synthesis in macrophages (Piquet et al., 1990; Zhang et al., 1993). It may also be noted several studies have indicated that  $\text{TNF-}\alpha$  is capable of stimulating ROS production from cellular systems (Zimmerman et al., 1989; Kumaratilake et al., 1990; Wang et al., 1999). Therefore ROS stimulated by  $\text{TNF-}\alpha$  may also play a role in cellular injury in response to inhalation of wood smoke

particles. Elevated TNF- $\alpha$  levels stimulate fibroblast proliferation and production of collagen matrix, leading to fibrosis (Brown et al., 1988b). It is possible that wood smoke may induce lung injury through induction of TNF- $\alpha$  release.

In conclusion, the results of the present study show that wood smoke is able to induce carbon-centered as well as reactive  $\cdot\text{OH}$  radicals and can in turn cause cellular damage. Wood smoke can cause lipid peroxidation, DNA damage, NF $\kappa$ B activation and TNF- $\alpha$  induction. It is proposed that  $\cdot\text{OH}$  radical plays an important role in these responses. Fe is present in wood smoke and  $\text{H}_2\text{O}_2$  can be generated in the respiratory burst of alveolar macrophages during phagocytosis of wood smoke particles. Thus, the reaction of Fe and  $\text{H}_2\text{O}_2$  could be a source of  $\cdot\text{OH}$  radicals in the lung. The results suggest that wood smoke is capable of causing acute lung injury and may have the potential to act as a fibrogenic agent.

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